PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :		(11) International Publication Number:
C08G 63/00, C12P 7/12, 7/62, C12N 1/12	A1	WO 97/07153
		(43) International Publication Date: 27 February 1997 (27.02.97)

(21) International Application Number: PCT/US95/10396

(22) International Filing Date: 14 August 1995 (14.08.95)

(71) Applicant *(for all designated States except US)*: UNIVERSITY OF MASSACHUSETTS MEDICAL CENTER [US/US]; 55 Lake Avenue North, Worcester, MA 01655 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: GROSS, Richard, A. [US/US]; 11 Ruthellen Road, Chelmsford, MA 01824 (US). SHI, Fengying [CN/US]; 830 Skyline Drive #22, Dracut, MA 01826 (US). ASHBY, Richard, D. [US/US]; 58 London Court, Merrimack, NH 03054 (US).

(74) Agent: FASSE, J., Peter; Fish & Richardson P.C., 225 Franklin Street, Boston, MA 02110-2804 (US).

(81) Designated States: AU, CA, CN, JP, KP, KR, NZ, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: METHODS OF CONTROLLING MICROBIAL POLYESTER STRUCTURE

(57) Abstract

The addition of PEG to culture media of *Alcaligenes eutrophus* and *A. latus* resulted in the following: (1) the controlled decrease in polyhydroxyalkanoate (PHA) molecular weight, which decreases the melt viscosity and bioresorption time; (2) the modulation of the repeat unit composition of the PHA products containing 3-hydroxybutyrate, 3-hydroxyvalerate, and 4-hydroxybutyrate, which provides polymers with varied physical properties; (3) the alteration of PHA repeat unit sequence distribution so that complex polymeric mixtures are obtained in place of random copolymers; and (4) the formation of PHA-PEG diblock copolymers where the carboxylate terminus of PHA chains are covalently linked by an ester bond to PEG chain segments. This is an example of the cellular production of a naturally synthesized diblock copolymer. The invention features new diblock copolymers, copolyester blends, and methods of preparation.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	**GB**	United Kingdom	**MW**	Malawi
AT	Austria	**GE**	Georgia	**MX**	Mexico
AU	Australia	**GN**	Guinea	**NE**	Niger
BB	Barbados	**GR**	Greece	**NL**	Netherlands
BE	Belgium	**HU**	Hungary	**NO**	Norway
BF	Burkina Faso	**IE**	Ireland	**NZ**	New Zealand
BG	Bulgaria	**IT**	Italy	**PL**	Poland
BJ	Benin	**JP**	Japan	**PT**	Portugal
BR	Brazil	**KE**	Kenya	**RO**	Romania
BY	Belarus	**KG**	Kyrgystan	**RU**	Russian Federation
CA	Canada	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CF	Central African Republic			**SE**	Sweden
CG	Congo	**KR**	Republic of Korea	**SG**	Singapore
CH	Switzerland	**KZ**	Kazakhstan	**SI**	Slovenia
CI	Côte d'Ivoire	**LI**	Liechtenstein	**SK**	Slovakia
CM	Cameroon	**LK**	Sri Lanka	**SN**	Senegal
CN	China	**LR**	Liberia	**SZ**	Swaziland
CS	Czechoslovakia	**LT**	Lithuania	**TD**	Chad
CZ	Czech Republic	**LU**	Luxembourg	**TG**	Togo
DE	Germany	**LV**	Latvia	**TJ**	Tajikistan
DK	Denmark	**MC**	Monaco	**TT**	Trinidad and Tobago
EE	Estonia	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**UG**	Uganda
FI	Finland	**ML**	Mali	**US**	United States of America
FR	France	**MN**	Mongolia	**UZ**	Uzbekistan
GA	Gabon	**MR**	Mauritania	**VN**	Viet Nam

Methods of Controlling Microbial Polyester Structure

Statement as to Federally Sponsored Research

This invention was made in part with Government support under grant No. DMR-9057233, awarded by the National Science Foundation. The Government has certain rights in the invention.

Background of the Invention

This invention relates to the control of the structure of microbially-produced polyester compositions such as polyhydroxyalkanoates.

Polyhydroxyalkanoates (PHAs) are a series of optically active, thermoplastic, water insoluble polyesters of alkanoic acids produced by various microorganisms. Since natural microbial PHAs are synthesized in aqueous media from renewable resources to form biodegradable thermoplastics, this process for polymer synthesis is an "environmentally friendly" preparative route. The microbial synthesis also avoids the use of organic solvents and toxic chemicals required for the chemical synthesis of PHAs. Also, since these microbial polyesters are biodegradable, they can be disposed of as part of the biowaste fraction of municipal solid waste.

The first member of the PHA family to be identified was poly(3-hydroxybutyrate), also known as "P3HB." See, e.g., Lemoigne, *Ann. Inst. Pasteur (Paris)*, 39:144, 1925; Lemoigne, *Bull. Soc. Chim. Biol.*, 8:770, 1926; and Lemoigne, Ann. *Inst. Pasteur (Paris)*, 41:148, 1927. A problem associated with P3HB is that melt-crystallized and solution-cast films of P3HB show brittle behavior which increases upon aging at room temperature.

PHAs with improved physico-mechanical properties have been created by incorporating different structural repeat units into PHAs. Over 50 different structural repeat unit types have been incorporated into PHAs to produce a large range of homo- and copolyesters. This structural diversity has been achieved by using different microbial production systems and by varying media carbon sources. These carbon sources are metabolized into hydroxyalkanoate repeat units having variable pendant group structures and number of carbon atoms between ester linkages.

Selected examples include poly[3HB-3-hydroxyvalerate-co-3-hydroxyhexanoate] (also referred to as "P[3HB-3HV-co-3HH"), described in Brandl et al., *Int. J. Biol. Macromol.*, 11:49 (1989); P[3HB-co-3HH] described in Shiotani et al., Japanese Pat. Appl. 93049 (1993), and Shimamura et al., *Macromolecules,* 27:878-880 (1994); and P3HV described in Steinbüchel et al., *Appl. Microbiol. Biotechnol.*, 39:443-449 (1993).

3-Hydroxyalkanoates that contain n-alkyl side groups with lengths generally from propyl to nonyl have also been produced, for example with functional side chain substituents such as phenyl and cyanophenoxy groups. A number of PHAs have also been reported that contain 4-hydroxbutyrate (4HB) repeat units, such as P[3HB-co-4HB] described in Kunioka et al., *Polym. Commun.*, 29:174 (1988) and Kunioka et al., *Appl. Microbiol. Biotechnol.*, 30, 569 (1989), and terpolyesters of 3HB, 3HV, and 4HB, described in Kimura et al., *Biotechnol. Lett.*, 14(6):445-450 (1992). In addition, 3-hydroxy-propionate (3HP) and 4HB repeat units have been found in PHAs produced by the bacterium *Alcaligenes eutrophus* (see, Kunioka et al., *Polym. Commun.*, 29:174-76, 1988, and Nakamura et al., *Macromol. Reports,* A28(Suppl.1):15, 1991).

Control of composition for copolyesters of 3HB and 4HB is normally achieved by variation in the carbon sources used or by alteration of other physiological parameters such as the incubation time and nitrogen concentration. For example, see, Nakamura et al., *Macromolecules*, 25, 4237-4241 (1992), and Doi, Y., *Microbial Polyesters*, VCH, New York (1990). In this way, PHAs can be chemically tailored to exhibit the desired physical-mechanical properties, crystallization rates, optical clarity, rheological properties, and biodegradation rates.

Thus, a variety of carbon source substrates have been used to form novel PHAs. However, known microbial synthesis methods provide no rational strategies to control polymer molecular weight or end group structure during the microbial polymerization.

Summary of the Invention

The invention is based on the discovery that when polyethylene glycol (PEG) of a known molecular weight is added to the culture medium of the bacterium *Alcaligenes eutrophus* or *Alcaligenes latus*, the structure of the resulting product can be controlled. For example, when *A. eutrophus* is placed in 4.0% PEG-200 supplemented media under polymer producing conditions, PEG-200 interacts with enzyme systems involved in PHA biosynthesis to cause dramatic product structural modulation. The cells respond to the PEG external stimulus by accumulating large quantities of oligomeric PEG that has a number average molecular weight (M_n) closely resembling that of the PEG added to cultivation media.

Specifically, addition of PEG-200 to culture media resulted in the following: (1) the controlled decrease in PHA molecular weight, which decreases the melt viscosity and bioresorption time; (2) the modulation of

the repeat unit composition of the PHA products containing 3HB, 3HV, and 4HB, which provides polymers with varied physical properties; (3) the alteration of PHA repeat unit sequence distribution so that complex polymeric mixtures are obtained in place of random copolymers; and (4) the formation of PHA-PEG diblock copolymers where the carboxylate terminus of PHA chains are covalently linked by an ester bond to PEG chain segments. This is an example of the cellular production of a naturally synthesized block copolymer.

In general, the invention features a method for producing a PHA having a controlled, e.g., decreased, molecular weight by culturing a PHA-producing microorganism, e.g., *Alcaligenes*, in a polymer production medium under conditions that allow the microorganism to produce a PHA, and adding PEG to the polymer production medium in an amount sufficient for the microorganism to produce a PHA having a molecular weight that is decreased relative to the molecular weight of a PHA produced by the same microorganism under the same growth conditions without PEG.

As used herein, a "polymer production medium" is used, e.g., for the second-stage fermentation, and includes a desired carbon source, but has a deficiency in one or more nutrients, e.g., nitrogen, oxygen, sulfur, or phosphate, that induces the microorganism to produce PHAs. These media are well known in the fermentation arts.

When *A. eutrophus* is used, the PEG can be added to the polymer production medium at a concentration of, e.g., 0.25 to 10.0 percent (weight/volume). When *A. latus* is used, the PEG can be added to the polymer production medium at a concentration of, e.g., up to 6.0 percent (weight/ volume).

In another embodiment, the invention features a method for incorporating 3-hydoxyvalerate (3HV) repeat units into a PHA using a non-3HV carbon source, e.g., 4-hydroxybutyric acid or 4-hydroxybutyrate, by culturing a PHA-producing microorganism in a polymer production medium containing a non-3HV carbon source under conditions that allow the microorganism to produce a PHA, and adding PEG to the polymer production medium in an amount sufficient for the microorganism to produce a PHA comprising 3HV, e.g., at a concentration of from 1 to 4 percent (weight/volume).

The invention further features a method for producing a PHA comprising a copolyester blend of at least two component polymers wherein each component polymer represents at least 30 percent by weight of the total blend, each component polymer is composed of at least 70 percent of a specific repeat unit structure, and the major repeat unit structure in each component polymer is different. This method is carried out by culturing a PHA-producing microorganism in a polymer production medium containing a carbon source, e.g., 4-hydroxybutyrate, under conditions that allow the microorganism to produce a PHA, and adding PEG to the polymer production medium in an amount sufficient for the microorganism to produce a PHA comprising a copolyester blend, e.g., at a concentration of 4 percent (weight/volume).

The invention also features a method for producing a polyhydroxyalkanoate-polyethylene glycol (PHA-PEG) diblock copolymer in which the carboxyl terminus of a PHA chain segment is covalently linked by an ester bond to a PEG chain segment by culturing a PHA-producing microorganism in a polymer production medium under conditions that allow the microorganism to produce a PHA, and adding PEG to the polymer production medium in an

amount sufficient for the microorganism to produce a PHA-PEG diblock copolymer.

For example, the polymer production medium can include glucose as the carbon source, and the microorganism can be *A. latus*. Then PEG can be added at a concentration of up to 6 percent (weight/volume). This method can be used to produce a PHA chain segment containing only P3HB repeat units. In addition, the polymer production medium can include 4-hydroxybutyric acid as the carbon source, and the microorganism can be *A. eutrophus*. Then the PEG can be added at a concentration of, e.g., 4 percent (weight/volume). This method can be used to produce a diblock copolymer comprising a majority of 4HB repeat units.

In another embodiment, the invention features a method for increasing the 4-hydroxybutyrate (4HB) mol percent in a PHA by culturing a PHA-producing microorganism in a polymer production medium containing 4-hydroxybutyric acid as a carbon source under conditions that allow the microorganism to produce a PHA, and adding PEG to the polymer production medium in an amount sufficient for the microorganism to produce a PHA of increased 4HB mol percent, e.g., 1 or 2 percent (weight/volume).

In another aspect, the invention features a PHA copolyester blend including first and second polymers each comprising at least 30 percent by weight of the blend, wherein the first polymer comprises at least 70 mol percent of a first repeat unit structure, the second polymer comprises at least 70 mol percent of a second repeat unit structure, and wherein the first and second repeat unit structures are different. For example, the first repeat unit structure can be 3-hydroxybutyric acid, the first polymer can comprise at least 90 mol percent of a first repeat unit structure, the second repeat unit

structure can be 4-hydroxybutyrate, the second polymer can comprise at least 80 mol percent of a second repeat unit structure.

The invention also features a polyhydroxyalkanoate-polyethylene glycol diblock (PHA-PEG) copolymer including a first chain of PHA repeat units and a second chain of PEG repeat units, wherein the second chain of PEG repeat units is covalently bound via an ester bond to a carboxy terminal end of the first chain of PHA repeat units. In particular examples, the first chain can be poly(3-hydroxybutyrate), and the second chain can have an average of 5 PEG repeat units, in which case the first chain can comprise an average of 220 PHA repeat units, or the first chain can comprise at least 80 mol percent of 4-hydroxybutyrate, and the second chain can have an average of 5 PEG repeat units, in which case the first chain can have an average of 435 PHA repeat units.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the detailed description, and from the claims.

Brief Description of the Drawings

Fig. 1 is a 500 MHz ^{1}H NMR Spectrum of purified PHA (*A. eutrophus*, carbon source (C.S.) 4-hydroxybutyrate, 4% PEG-200).

Fig. 2 is an expansion of a two dimensional homonuclear (^{1}H) correlated (COSY) spectrum of PHA (*A. eutrophus*, C.S. 4-hydroxybutyrate, 4% PEG-200).

Figs. 3a to 3c are a series of gel permeation chromatography (GPC) traces of products formed (3a:*A. eutrophus*, C.S. 4-hydroxybutyrate, 0% PEG-200, crude; 3b:*A. eutrophus*, C.S. 4-hydroxybutyrate, 4% PEG-200, crude; and 3c:*A. eutrophus*, C.S. 4-hydroxybutyrate, 4% PEG-200, one time precipitated).

Fig. 4 is a 125 MHz ^{13}C NMR spectrum of PHA (*A. eutrophus*, C.S. 4-hydroxybutyrate, 4% PEG-200).

Figs. 5a to 5c are a series of expanded 75 MHz ^{13}C NMR spectra for carbonyl resonances of PHA (5a:*A. eutrophus*, C.S. 4-hydroxybutyrate, 4% PEG-200; 5b:Acetone soluble (AS) fraction of sample 5a; and 5c:Acetone insoluble (AIS) fraction of sample 5a).

Figs. 6a to 6c are a series of differential scanning calorimetry (DSC) thermograms of PHAs (First Heating)(6a:*A. eutrophus*, C.S. 4-hydroxybutyrate, 4% PEG-200; 6b:Acetone soluble fraction of sample 6a; and 6c:Acetone insoluble fraction of sample 6a).

Figs. 7a to 7c are a series of DSC thermograms of PHAs (Second Heating) (7a:*A. eutrophus*, C.S. 4-hydroxybutyrate, 4% PEG-200; 7b:Acetone soluble fraction of sample 7a; and 7c:Acetone insoluble fraction of sample 7a).

Fig. 8 is a graph showing the effects of PEG-200 media concentration on the number average molecular weights (M_n) of the resulting PHAs from *A. eutrophus* and *A. latus*.

Detailed Description

Use of PEG to Control PHA Structure

PEG with M_n of about 200 g/mol (PEG-200) was added in an amount up to 10% (w/v) to cultivations of *A. eutrophus* and 6% to cultivations of *A. latus*, either initially or during the polymer production stage, e.g., the second stage of a two stage fermentation, to study: (1) the effect of PEG-200 on the conversion by *A. eutrophus* and *A. latus* of the carbon source 4-hydroxybutyrate (4HB) to polyester, (2) changes in product molecular weight, and (3) the incorporation of PEG chain segments that are covalently linked to microbial polyester products.

Gel permeation chromatography (GPC) was used to investigate product molecular weight averages and dispersity. One- and two- dimensional ^1H nuclear magnetic resonance (NMR) spectroscopy were used to study the repeat unit composition and incorporation of PEG-200 in various product fractions. ^{13}C NMR spectroscopy was used to analyze polymer repeat unit sequence distribution. Fractionation by differential solubilities in acetone was used to investigate product heterogeneity. In addition, differential scanning calorimetry (DSC) was used to obtain information on thermal transitions of products and product fractions.

Polyethylene Glycol

PEG-200 (200 g/mol) used in these studies was purchased from Aldrich. The PEG number average molecular weight (M_n) was confirmed by using ^1H NMR end group analysis and was found to be 194 g/mol.

Bacterial Preservation and Inoculum Preparation

Alcaligenes eutrophus (ATCC 17699) was used in this study. This strain was first grown under aerobic conditions as described in Ervine, Chap. 2 in Fermentation, A Practical Approach (McNeil et al. (eds.),

IRL Press, 1990) at 30°C for 14 hours, the culture was then diluted with 2 parts of 20% glycerol and transferred into 1 mL cryogenic vials. The vial contents were frozen in a dry ice-ethanol bath and then stored in liquid nitrogen. The cells contained in the vials were used as the inoculum for the two-stage fermentation reactions described below.

Alcaligenes latus (DMS 1122) was also used in the methods of the invention.

A. eutrophus Fermentation Conditions

100 mL Cultivations (Cultivation Condition A): A nutrient rich medium (100 mL, as described in Kunoika et al., *Appl. Microbiol. Biotech.*, 30:569, 1989) was prepared, autoclaved to sterilize, and inoculated with 0.1 mL cells from a thawed cryovial. *A. eutrophus* was grown in 500 mL baffled Erlenmeyer flasks in a shaker incubator at 30°C, 250 RPM, for 24 hours. The cells were harvested by centrifugation (4°C, 8,000 rpm for 20 minutes) and washed with a sterile Na_2HPO_4-NaH_2PO_4 buffer solution at pH 7.0. Typically, the cell dry weight of these first stage cultivations was 0.5 g/L. The washed cells were then transferred under aseptic conditions into 100 mL of a sterile filtered nitrogen-free medium which contained 1.51 g/L Na_2HPO_4, 2.65 g/L KH_2PO_4, 0.2 g/L $MgSO_4$, 1.0 mL/L Microelement solution (Kunioka et al., *Appl. Microbiol. Biotechnol.*, 30, 569, 1989), 4-hydroxybutyric acid (1.5 g) or fructose (1.5 g), and either 0, 1, 2, or 4% (wt/vol) PEG-200. Polymer production was then carried out by cultivation of *A. eutrophus* in the above media using a 500 mL Erlenmeyer flask at 30°C, 250 RPM, for 48 hours. The cells were then separated by centrifugation, washed with about 10 mL of water per gram of wet cells, and lyophilized.

500 mL Cultivations (Cultivation Condition B): Increased PHA from media amended with 4% PEG needed for

fractionation and subsequent analysis (see below) was obtained as described above by the two-stage method but using 2800 mL Erlenmeyer flasks and 500 mL cultivation volumes.

A. latus Fermentation Conditions

A. latus was grown on 1.0% glucose (w/v) in a one-stage fermentation where PEG-200 was added to the initial growth media as described in Hiramitsu, et al., *Biotechnol. Letters* **15**:461 (1993). It was anticipated that under these conditions, the PHA formed by *A. latus* would be P3HB. *A. latus* grew and divided initially in the presence of up to 3% (w/v) PEG-200, but showed no bacterial growth, and hence no PHA production at 4% PEG-200. Increasing the media concentration of PEG-200 from 0 to 1% caused little change in the cell and polymer yields. However, at a media concentration of 3% PEG-200, the biomass and polymer yield productivity dropped precipitously.

Polymer Isolation

The intracellular PHAs formed from each *Alcaligenes* species were extracted from cells by stirring a suspension of lyophilized cells (about 0.5 g) for 48 hours in chloroform (80 mL) at room temperature. The insoluble cellular material was removed by filtration, and the solvent was then evaporated to obtain what is termed herein the "crude product." Precipitated products were isolated by concentrating the chloroform crude product solution to a total volume of ~4 mL and precipitation of the polymer in 30 mL of methanol. The resulting precipitate was washed with methanol and ether and then dried *in vacuo*. Unless otherwise specified, the isolated products were obtained using one precipitation/washing cycle.

The PHA formed from 4-hydroxybutyrate in the medium with 4% PEG (cultivation condition B, see above)

and isolated by one precipitation/washing cycle was dissolved in chloroform (0.1 g/mL). Acetone (10 volumes) was slowly added to the chloroform solution. The white cotton-like precipitate which resulted from acetone addition was isolated by filtration giving the acetone insoluble (AIS) fraction. The solvent was evaporated from the acetone-chloroform solution which gave the acetone soluble (AS) fraction. Removal of residual solvents from the AS and AIS fractions was carried out in a vacuum dessicator (10 mm Hg, 24 hours) and the samples were then allowed to age for at least one week at ambient temperature prior to carrying out thermal analyses.

Polymer Characterization

A UNITY-500 NMR Spectrometer was used for 1 and 2-D proton NMR experiments described below. Proton (^{1}H) NMR were recorded at 500 MHz. Chemical shifts in parts per million (ppm) were reported downfield from 0.00 ppm using tetramethylsilane (TMS) as an internal reference. The experimental parameters were as follows: 0.5% w/v polymer in chloroform-d, temperature 298°K, 2.4 μsec (14°) pulse width, 3 second acquisition time, and 6,000 Hz spectral width. Carbon (^{13}C) NMR spectra were recorded using a Varian XL-300 at 75.4 MHz and the following parameters: 2.0% w/v polymer in chloroform-d, 298°K, 9.7 μsec pulse width, 1 second acquisition time and 2 second pulse delay, 16502 spectral width, 33024 data points, and 14400-19600 accumulations. The observed ^{13}C NMR chemical shifts in ppm were referenced relative to chloroform-d at 76.91 ppm. For the COSY experiment (0.5% w/v polymer in chloroform-d) the data were collected in a 1024 x 256 data matrix and zero-filled to 1024 x 1024 using 8 scans per increment, a 4260 Hz sweep width, and a 1.1 second delay between transients. The data was processed using sinebell weighting.

The polymers produced by *A. latus* were analyzed by 1-D-^{1}H NMR (500 MHz) and COSY (500 MHz) spectroscopy. Spectra of PHA samples were recorded on a Varian XU-500 spectrometer. Parameters for the 1-D-^{1}H and COSY polymer spectra were as follows. 1.0% (w/v) polymer in $CDCl_3$, temperature 298°K, 2.4 and 14.5 μsec pulse widths, 8000 and 2710 spectral widths, 3.0 and 0.189 second acquisition times, 0 and 1.0 second delay times, and 45 and 8 transients, respectively.

The molecular weights of polyesters were determined by GPC studies using a Waters HPLC system with 500-, 10^3-, 10^4-, and 10^5-Å Ultrastyragel columns placed in series. Chloroform (HPLC grade) was used as the eluent at a flow rate of 1.0 mL/min, sample concentrations were typically 3 to 10 mg/mL and the injection volume was 100 μL. Detection was by refractive index (Waters Model 410). Polystyrene standards (Aldrich) with low polydispersities were used to generate a calibration curve from which product molecular weights were determined with no further corrections.

All thermal characterizations were carried out using a DuPont 2910 differential scanning calorimetry (DSC) equipped with a TA 2000 data station, using between 5.0 to 6.0 mg of sample sealed in aluminum pans and a dry nitrogen purge. The polymer samples were heated at a rate of 10 °C/min from room temperature to 200°C, rapidly quenched from the melt and then were analyzed during second heating scans from -80°C to 200°C. Data reported for the melting temperature(s), T_m, and enthalpy of fusion(s), ΔH_f, were taken from the first heating scan. The reported glass transition temperature (T_g) values were the midpoint values measured during the second heating scans.

EXAMPLES

Effect of PEG on PHA Repeat Unit Composition

Control of composition for copolyesters of 3HB and 4HB is normally achieved by variation in the carbon sources used or by alteration of other physiological parameters such as the incubation time and nitrogen concentration. In this study, PEG-200 was added to *A. eutrophus* cultivations in concentrations up to 4% (w/v) during the second or polymer producing stage of the fermentation where 4HB served as the carbon source. The mol fractions of repeat units for PHAs isolated by one precipitation/washing cycle were analyzed by ^{1}H NMR spectral integration of well resolved signal regions (see Fig. 1) as has been previously described, e.g., in Nakamura et al., *Macromolecules*, **25**:4237-4241 (1992).

When *A. eutrophus* is grown on 4HB without PEG added, the resulting PHA closely approximates a random copolyester of 3HB and 4HB repeat units of high molecular weight, with no 3HV or EG repeat units (see Table I, below). Upon the addition of PEG-200 in culture conditions A, dramatic shifts in the repeat unit composition were achieved. Table I below shows the effects of PEG-200 on the production and compositions of microbial polyesters formed by *A. eutrophus* using 4HB as carbon source. In particular, the mol% of 4HB in the product changed from 66% with 0% PEG-200 added, to 86% with 2% PEG-200 added. Upon further addition of PEG-200 from 2 to 4%, the mol% of 4HB decreased. Furthermore, the addition of PEG-200 resulted in products containing low level incorporation of 3HV repeat units (see Table I).

Table I

polym, %PEG,	cult cond	cell yield, g/L	polym. content of cells, %	PHA yield, g/L	repeat units found in PHA mol %				M_n, g/mol X 10^{-3}	M_W/M_n
					3HB	4HB	3HV	EG		
0	A	3.7	21	0.76	34	66	0	0	222.1	2.76
1	A	3.5	16	0.56	20	79	1.1	0	178.6	1.89
2	A	3.1	14	0.45	11	86	2.8	0.28	153.0	2.05
4	A	2.6	14	0.37	30	64	5.0	0.93	112.2	2.51
0	B	3.9	27	1.1	70	30	0	0	198.6	2.87
4	B	3.7	26	0.97	41	53	5.4	1.1	77.2	3.95
4-AS	B				13	84	2.1	1.6	37.4	2.52
4-AIS	B				95	3	2.0	0.1	130.0	3.42

In Table I, PEG was added to the cultivation medium during the second, polymer producing stage. Non-fractionated samples were obtained from one precipitation/ washing cycle. As described in further detail below, 4-AS and 4-AIS are the acetone soluble (57% w/w) and insoluble (43%) fractions of the 4% PEG product obtained using cultivation condition B. The cell yield is the quantity of harvested cells after they were washed with nanopure water and lyophilized. The polymeric content of the cells is expressed as the percent of the cellular dry weight which contains PHA. These values were obtained gravimetrically from the isolated product from chloroform extraction and one precipitation/washing cycle. The PHA yield is the (cell yield) X (fraction of the cellular dry weight which is PHA). The M_n and M_W/M_n were determined by GPC. The cultivation conditions are either condition A or B as indicated. This experiment was repeated and the identical trends were observed.

As shown in Table I, small quantities of PEG-200 added to fermentation media caused important product

As shown in Table I, small quantities of PEG-200 added to fermentation media caused important product compositional changes. The addition of PEG to the media increased both 4HB and 3HV contents, while decreasing the 3HB contents. It is known, e.g., as described in Doi et al., Microbial Polyesters (VCH Publishers, N.Y. 1990), that by decreasing the mol percentage of 3HB while increasing the relative contents of 4HB or 3HV in polymers, products are formed that have a relatively higher flexibility and elongation at break.

Formation of PHA-PEG Diblock Copolymers

The ^{1}H NMR spectrum of the PHA isolated by one precipitation/washing cycle for a cultivation containing 4% PEG-200 (culture condition B, see Table I) is shown in Fig. 1. Weak ^{1}H NMR signals at ~3.7 ppm were observed that correspond to protons (a,c,d,e) of ethylene glycol (EG) repeat units. In contrast, P(3HB-co-4HB) formed in the absence of PEG (not shown) does not show any ^{1}H NMR signals in the 3.6 to 3.8 ppm spectral region.

A COSY spectrum of this product was recorded and the specific spectral regions of interest are shown in Fig. 2. Three ^{1}H signals at 4.25, 4.35, and 4.46 ppm were observed that have correlations (coupling between neighboring ^{1}H nuclei) with signals at 3.70, 3.73 and 3.77 ppm, respectively. The signal at 4.25 ppm also has a contribution from a satellite peak of protons 8 (4.1 ppm) (in Fig. 1) due to ^{13}C-^{1}H coupling. Based on chemical shift parameters documented for model compounds, it is expected that esterification of a terminal PEG-CH_2-OH will lead to a downfield shift from ~3.7 to ~4.25 ppm.

Considering these results and data, the signals in the 3.68 to 3.80 and 4.20 to 4.50 ppm regions were assigned to protons a and b, respectively, of esterified PEG chain segments. Correlation of the signals with

peaks at 3.62 and 3.73 suggest that they are due to protons e and d of terminal free hydroxyl EG units (see Fig. 2). Assuming that the contribution of the overlapping signals in the 3.6 to 3.8 region can be estimated by Bernoullian curve fitting, the area under peaks was measured by cutting and weighing. The integration results showed that the ratio of protons a + d to c to 2x e was 3:6:2. Using the ratio c to a + d and c to 2x e gives values of n (internal EG units of 2 and 3, respectively).

Thus, the average chain length of PEG segments in the diblock copolymer is between 4 and 5 which corresponds to molecular weights of ~180 and 220 g/mol, respectively. The above results are consistent with the formation of PHA chains that are covalently linked at the carboxylate chain terminus to PEG chain segments, which indicates that PHA-PEG diblock copolymers were formed (see Figs. 1 and 2). Furthermore, the average PEG chain length in the product is almost identical to that which was provided in the cultivation media.

Such PHA-PEG diblock copolymers include a long PHA chain segment (average of 430 4HB repeat units) that is covalently linked with an ester bond at its carboxy terminal end to a relatively short PEG chain segment (average of 5 repeat units). These PHA-PEG diblock copolymers provide unique characteristics compared to PHA products currently available. For example, the diblock copolymers include terminal ethylene glycol (EG) hydroxyl functionalities that allow the formation of chemical linkages with drugs, they have amphipathic characteristics, and they can be used in blends as compatibilizing agents.

The invention also provides a unique method to incorporate PEG into PHA formulations such that the PEG will leach out of the PHA into an aqueous media at a much

slower rate than formulations in which PHAs and PEG are merely mixed together.

The following experiments were performed to provide further evidence that ^{1}H NMR signals observed in the 3.6-3.8 ppm region for one-time-precipitated products were not due in part to residual PEG-200. P(3HB-co-30% 4HB) (produced by a cultivation of *A. eutrophus* with no added PEG-200) and PEG-200 (286 and 218 mg, respectively) were dissolved in chloroform and cast to form a film. This film contained 43% by weight PEG-200, which exceeds by a factor of ~2 times the quantity of PEG-200 found in the corresponding crude product (non-precipitated-solution extracted material, see discussion below). The film was then purified by one precipitation/washing cycle using identical conditions as was used for isolated products. The resulting isolate contained 0 mol % PEG based on ^{1}H NMR analysis. Therefore, no residual PEG exists in the one-time-precipitated product.

In addition, PEG-200 (0.3% w/w) was mixed with a PHA-PEG product (0.7% w/w) obtained after 3 precipitation/ washing cycles from 4% PEG amended cultivations. Once again, after only one precipitation/washing cycle, the relative signal intensities of the 3.6-3.8 ppm signal region to PHA protons was identical to that of the PHA-PEG product prior to mixing with PEG-200. Moreover, repeated precipitation/washing (up to three times) of one-time-precipitated samples did not result in a change in the EG mol percentage. Therefore, non-covalently linked PEG-200 is indeed removed efficiently from the isolated products by one precipitation/washing cycle.

Formation of Copolyester Blends

PHAs isolated from *A. eutrophus* cultivations in which 4-hydroxybutyric acid served as a carbon source and

PEG-200 was not added to the media have sequence distributions of 3HB and 4HB repeat units that are approximately random. See, e.g., Nakamura et al., *Macromolecules*, 25:4237-4241, 1992.

A GPC trace of the extracted crude material from *A. eutrophus* cultivations (culture condition B using 4-hydroxybutyrate as the carbon source, see Table I) containing 4% PEG was quite complex indicating that it is a mixture or blend of polymers having very different molecular weight averages (see Fig. 3b). The mixture has unique physical and biological properties. In contrast, the GPC trace of the crude polymer product obtained from cultivation media without PEG-200 shows only a unimodal peak (see Fig. 3a). Also, the GPC trace of the crude product has a component peak with an elution volume which corresponds exactly with that of PEG-200 (Fig. 3b, peak at 200 g/mol). This is further evidence that PEG-200 does indeed accumulate in the cells, and that this occurs without notable cellular selectivity as a function of PEG chain length.

Consistent with the studies above in which PEG-200 was mixed with PHAs and removed by one precipitation/washing cycle, the GPC trace of the one-time-precipitated product (Fig. 3c) shows no trace of residual PEG-200, but still shows multiple component peaks.

Fractionation of PEG Cultivation Products

The PHA product was fractionated based on its solubility in acetone. Fractionation resulted in an acetone soluble fraction (AS) representing 57% (w/w) of the total product, and an acetone insoluble fraction (AIS) representing 43% (w/w) of the total product. The AS and AIS fractions had M_n (M_w/M_n) values of 37,400 (2.52) and 130,000 (3.42), respectively (see Table I). The fact that the PHA product could be fractionated

provides additional evidence that the product is a mixture or blend of polyesters as opposed to a block copolymer.

Analysis of the repeat unit composition of these fractions by ^{1}H NMR spectral integration showed that the mol fractions of 3HB, 4HB, 3HV, and EG repeat units are 13, 84, 2.1, 1.6 and 95, 3, 2, 0.1, respectively (see Table I). Thus, the addition of PEG-200 to cultivation media results in the formation of a new product that is a blend of polyesters in contrast to the random copolymers formed in the absence of PEG-200.

Also, the PEG chain segments are found primarily in the AS high 4HB fraction (see Table I). This is evidence that for *A. eutrophus*, linkages between PEG and PHA segments occur primarily between 4HB and EG repeat units.

Using a model in which it is assumed that PEG segments are at all carboxyl terminal positions of PHA chains, the M_n calculated molecular weight based on ^{1}H NMR spectral integration is 24,000 g/mol, whereas the experimentally determined value from GPC is 37,400 g/mol. From this analysis, the results are consistent with this model.

NMR Analysis of the Copolyester Blend Fractionations

The effects of PEG-200 on the repeat unit sequence distribution were studied using the ^{13}C NMR spectra for the one time precipitated/washed products obtained from fermentations with 0 and 4% PEG-200 (culture condition B). The ^{13}C NMR spectrum for the latter product is shown in Fig. 4 and expansions of the carbonyl regions of the unfractionated product, acetone soluble (AS) fraction, and acetone insoluble (AIS) fraction are shown in Figs. 5a, 5b, and 5c, respectively. The assignments of the observed signals, including those in the carbonyl region

which are sensitive to effects of repeat unit sequence distribution, were made as described in, e.g., Nakamura et al., *Macromolecules*, **25**:4237-4241 (1992).

To simplify the repeat unit sequence analysis below, the small contributions from 3HV and EG repeat units were neglected so that the products were assumed to consist of only 3HB and 4HB repeat units. The relative mol fractions of 3HB*-3HB (3*3), 3HB*-4HB (3*4), 4HB*-3HB (4*3) and 4HB*-4HB (4*4) dyads (see Figs. 5a to 5c) were determined by spectrometer integration and are given in Table II below, which shows the experimental and calculated comonomer dyad fractions for PHAs and product fractions formed in cultivations with and without PEG-200.

Experimental values were compared to those calculated assuming a Bernoullian or random statistical process for microbial catalyzed copolymerization using the following relationships (equations 1-3) where F_3 is the mole fraction of 3HB units in the polymer as described, e.g., in Doi et al., *Macromolecules*, **21**:2722 (1988):

$$[3*3] = F_3^2 \quad (1)$$

$$[3*4] = [4*3] = F_3(1-F_3) \quad (2)$$

$$[4*4] = (1-F_3)^2 \quad (3)$$

Table II

polym % PEG,	Dyad Sequence 3HB-3HB exp (calcd)	3HB-4HB exp (calcd)	4HB-3HB exp (calcd)	4HB-4HB exp (calcd)
0	0.60 (0.49)	0.14 (0.21)	0.10 (0.21)	0.16 (0.096)
4	0.57 (0.32)	0 (0.25)	0 (0.25)	0.43 (0.18)
4-AS	0.07 (0.03)	0.14 (0.15)	0.14 (0.15)	0.66 (0.67)
4-AIS	1.0 (0.85)	0 (0.07)	0 (0.07)	0 (0.01)

In Table II, each column shows the experimental values (exp), determined by measuring the relative peak areas for the carbonyl carbon ^{13}C NMR signals assigned (see Figs. 5a to 5c) to the four dyad sequences, and the calculated value (calcd), determined from equations 1 to 3, assuming a Bernoullian or random statistical process and that the contribution of 3HV and EG repeat units can be neglected. In the first column, the indicated percent PEG was added to the cultivation medium during the second or polymer producing stage. The non-fractionated sample was obtained from one precipitation/washing cycle from a cultivation carried out using 500 mL of media in a 2.8 L shake flask. The AS and AIS fractions of the 4% PEG product represent 57% and 43% (w/w) of the product, respectively.

Table II shows that the PHA produced with 0% PEG approximates a random copolyester. In contrast, the addition of 4% PEG to cultivations resulted in a novel product that has predominantly 3HB*-3HB and 4HB*-4HB dyads (see also, Table I rows 4-AS and 4-AIS, and Fig. 5a). Thus, the addition of PEG to the growth medium provides a new microbial polymerization process in which polymer blends are made directly by a single fermentation reaction. Therefore, this new process is much more

efficient than prior methods to produce blends of polymers in which one component polymer has a high (greater than 70%, and preferably greater than 90%) 3HB content, and the other component polymer has a high (greater than 70%, and preferably greater than 80%) 4HB content. Furthermore, each of the two component polymers represents at least 30% of the total weight of the blend.

Thermal Analysis of the Copolyester Blend Fractionations

Table III, below, shows the results of thermal analysis obtained by DSC measurements at a scanning rate of 10°C/min. The percent PEG added to the cultivation medium was added during the second or polymer producing stage. The non-fractionated samples were obtained from one precipitation/washing cycle. Again, 4-AS and 4-AIS are the acetone soluble (57% w/w) and insoluble (43%) fractions of the 4% PEG sample. In Table III, T_g represents glass transition temperatures taken as the midpoint of the heat capacity change and measured during the second heating scan after rapidly quenching by liquid nitrogen at -70°C from the melt. T_m represents the peak melting temperatures for each endothermic melting transition determined during the first heating scan. ΔH_f (cal/g) represents the heat of fusion value measured for each melting endothermic transition. Cultivations were carried out using 500 mL of media in a 2.8 L shake flask (culture conditions B).

Table III

polym, (% PEG w/v)	T_g (°C)	T_m (°C)	ΔH_f (cal/g)
0	3 -15	165	9.9
4	-45 -29	55 170	5.0 11.6
4-AS	-42 -15	50	8.8
4-AIS	2.8	172	19.0

As shown in Table III, the DSC thermograms of the 4% PEG product during a first heating scan showed two distinct T_m values at 55° and 170°C (see also Fig. 6a) which closely approximate reported T_m values for P3HB and P4HB (177° and 54° C, respectively). The DSC thermogram of this product recorded during a second heating scan after rapidly quenching from the melt showed T_g values at -45° and -29°C (see also Fig. 7a). The T_g at -45°C closely approximates that reported for P4HB (-50°C) while the T_g at -29°C is intermediate to those reported for P3HB (~4°C) and P4HB. This indicates the formation of a copolymer blend of predominantly P3HB and P4HB. The observed T_g at -29°C may result from the formation of a small product fraction that consists of random 3HB/4HB copolyester chains having the corresponding T_g value. In contrast, the product obtained from cultivations with no added PEG had T_m and T_g values of 165 and 3°C, respectively, which is consistent with the formation of a random copolyester.

^{13}C NMR and DSC measurements of the AS and AIS fractions were made to further characterize the individual component polymers of the copolyester blend formed in 4% PEG amended media. Expansions of the ^{13}C NMR carbonyl spectral regions for these fractions are shown in Figs. 5b and 5c, respectively. DSC thermograms of the

first and second heating scans are shown in Figs. 6a to 6c and 7a to 7c, respectively. If the T_g, T_m, and ΔH_f values for solution precipitated P3HB are taken as 4°C, 20.8 cal/g, and 177°C, respectively, comparison of these data to those obtained for the AIS fraction (see Table III) indicates that this fraction contains primarily P3HB homopolymer, as opposed to a random copolyester such as P(3HB-co-6 mol% 4HB) that has been shown to have T_m and ΔH_f values of 162°C and 13.5 cal/g, respectively (Nakamura et al., *Macromolecules*, **25**:4237-4241, 1992). This is further supported by the ^{13}C NMR spectrum of the AIS fraction which shows only 3HB*-3HB dyads (see Fig. 5c).

The dyad sequence distribution of the AS fraction determined experimentally (see Fig. 5b), and calculated using equations 1 to 3, above, suggests that the product formed approximates that of a high 4HB content random copolyester (see Table II). Further study of this fraction by DSC indicated product heterogeneity. Specifically, the AS fraction had multiple Tg (-15,-42°C) transitions and a broad melting region (see Table III, Figs. 6b and 6b). A comparison of the thermal transitions of this product fraction with those previously reported for 3HB/4HB random copolyesters (Nakamura, 1992, supra) indicates that the AS fraction is composed of P(3HB-co-90% 4HB) and P(3HB-co-28% 4HB) random copolyesters (T_g values of -44 and -15°C, respectively)(Nakamura, 1992, supra).

This analysis, assuming that the components are immiscible, indicates that the AS fraction is a mixture of random copolyesters with relatively high and low 4HB contents (~90-94 and ~30 mol%, respectively) with weight fractions of ~86 and 14%, respectively. Thus, it appears that the unfractionated product from media containing 4% PEG is indeed complex as was originally indicated by the

GPC trace (see Figs. 3b and 3c, above), and is composed of at least three different component polymers of different repeat unit composition.

Effect of PEG on PHA Molecular Weight and Yield

Results of Studies on *A. eutrophus* (C.S. 4-Hydroxybutyric Acid)

Table I also depicts the effects of PEG-200 on volumetric yield and product molecular weight for the series of fermentations of *A. eutrophus* carried out under culture conditions A. The volumetric yield of the PHAs continued to decrease with increased PEG media concentration so that for 2 and 4% PEG-200 addition the yields were approximately 59% and 49%, respectively, of that for PEG deficient media. The M_n and M_w/M_n values measured by GPC of the products formed from cultivations with 0, 1, 2, and 4% PEG are also shown in Table I. The GPC traces of these products were unimodal. An increase in the PEG concentration from 0 to 4 percent resulted in a decrease in product molecular weight (M_n) from 222,100 g/mol to 112,200 g/mol, e.g., about 50 percent. Thus, PEG can be used to form PHAs that contain 4HB repeat units and have reduced molecular weights compared to PHAs produced without PEG. A decrease in molecular weight affects the polymer characteristics, e.g., decreases the melt viscosity, and is useful to form sustained release compositions and biomaterials that require relatively shorter bioresorption times.

Comparison of PEG Effects on P3HB Molecular Weight in *A. latus* and *A. eutrophus*

PEG-200 was added to the fermentation media of each organism either initially, or at the beginning of the second stage of a two stage fermentation. Table IV shows the effects of PEG-200 on bacterial growth, polymer production, and polymer composition from 1 and 2 stage

cultivations of *A. latus* grown on glucose (designated by the letter L), and 2 stage cultivations of *A. eutrophus* grown on fructose (designated by the letter E). In Table IV, R-CDW is "residual cell dry weight" which corresponds to the non-polymer weight of the cells which may be considered the residual biomass.

Table IV

Product	Initial PEG-200 conc. (%), and incubation time (hr)	PEG-200 supplement final conc. (%), incubation time (hr)	Cell Yield (g/L)	Polymer Yield (g/L)	Cellular Productivity (mg/mg R-CDW)[a]	Compositions (mol%) [3HB (EG)]
Alcaligenes latus DSM 1122						
1 Stage Fermentation						
L-1	0 (48)	-	1.9	2.8	1.5	100(0)
L-2	1 (48)	-	1.8	2.7	1.5	98.2(1.8)
L-3	2 (48)	-	1.6	2.1	1.3	97.7 (2.3)
L-4	3 (48)	-	0.9	0.3	0.3	97.8 (2.2)
2 Stage Fermentation						
L-5	2(24)	4(24)	1.6	1.4	0.9	96.3 (3.7)
L-6	2(24)	5(24)	1.7	1.3	0.8	96.4 (3.6)
L-7	2(24)	6(24)	1.9	0.8	0.4	96.4 (3.6)
Alcaligenes eutrophus ATCC 17699						
2 Stage Fermentation						
E-1	0(24)	0(48)	3.3	3.0	0.9	100(0)
E-2	0(24)	0.25(48)	3.0	2.9	1.0	100(0)
E-3	0(24)	0.5 (48)	2.8	2.9	1.0	100(0)
E-4	0(24)	1(48)	2.7	2.7	1.0	100(0)
E-5	0(24)	1.5(48)	2.6	2.5	1.0	100(0)
E-6	0(24)	2(48)	2.9	2.4	0.8	100(0)
E-7	0(24)	5(48)	1.7	1.7	1.0	100(0)
E-8	0(24)	10(48)	2.3	0.3	0.1	100(0)

Results in *A. latus*

As shown in Table IV, increasing the media concentration of PEG-200 from 0 to 1% caused little change in the cell and polymer yields for *A. latus*. However, at a media concentration of 3% PEG-200, the biomass and polymer yield productivity dropped precipitously. These results are likely due to the increased osmotic stress caused by increasing media PEG-200 concentrations.

Further, the cellular productivity of *A. latus* was constant between 0 and 2% PEG-200 concentrations, but at 3% PEG-200, cellular productivity was reduced by 80% to 0.3 mg/mg R-CDW. This reduction indicated that above 2% PEG 200, the decrease in product yield was due not only to poorer cell yields, but was also the result of a less efficient production system.

The molecular weights of the isolated polymer products formed by *A. latus* were analyzed by gel permeation chromatography (GPC). Unless otherwise specified, the isolated products were obtained using 2 precipitation/ washing cycles.

The addition of only 1% PEG-200 to *A. latus* cultivation media resulted in a decrease in the M_n by 85% from 238,000 g/mol to about 35,000 g/mol (see Fig. 8). Further increases in the media PEG-200 concentration from 1 to 3% resulted in little to no further molecular weight change. Thus, low PEG-200 media concentrations (1%) can be used to modify product molecular weight.

For two-stage cultivations of *A. latus*, polymers were formed in production medium containing up to 6% PEG-200, where 2, 3, and 4% PEG were added to cultivations containing 2% PEG-200 after a first-stage 24 hour cultivation. This two-stage approach resulted in polymers with M_n values as low as 19,000 g/mol. Furthermore, products L-2 to L-7, when analyzed by 1- and

2-D proton NMR as described above for products derived from 4-hydroxybutyric acid, were shown to contain diblock copolymers of a P3HB chain segment covalently linked at its carboxyl terminal end to a PEG chain segment.

Results in *A. eutrophus*

For comparative purposes, a series of two-stage cultivations were carried out using *A. eutrophus* as the microbial production system where variable quantities of PEG-200 were added to cultivation media (see Table IV). Cellular growth and polymer production for all *A. eutrophus* cultures were carried out using a two-stage batch culture process as described above for cultivation conditions A, using fructose as the carbon source for the second stage cultivations.

A. eutrophus showed only small decreases in cell and product yield with the addition of up to 2% PEG-200. Also, the cellular productivity calculated using the non-polymer or residual cell dry weight (R-CDW, see Table IV) remained almost unchanged (~1.0 mg/mg R-CDW) for media containing up to 5% PEG-200, but decreased to 0.13 mg/mg non-polymer CDW when the PEG-200 media concentration was increased to 10%. Thus, *A. eutrophus* showed an excellent tolerance to the osmotic stress imposed by the solute PEG-200.

Fig. 8 shows that the molecular weight of product polyesters was decreased by increasing the media PEG-200 concentration. In fact, there was a regular decrease in product molecular weight as the PEG-200 concentration was increased from 0 to 1% (M_n values of 650,000 g/mol and 104,000 g/mol, respectively). As was observed for *A. latus*, further increases in the media PEG-200 concentration from 1 to 5% resulted in substantially less molecular weight reduction per added PEG increment (see Fig. 8). Thus, sensitive control of product molecular weight was achieved by variation of the media PEG-200

concentration from 0 to 1% for both *A. latus* and *A. eutrophus*.

USE

The methods described above allow the modification of microbial polyester products by simply changing the concentration of PEG added to the cultivation medium. By controlling the amount of PEG added, one can control the molecular weight, repeat unit composition and distribution, and produce specified copolyester blends as opposed to random copolyester chains.

In particular, such diblock copolymers and copolyester blends of the invention can be used to make biodegradable plastic articles and coatings, e.g., for paper, that are manufactured by standard thermal processing methods. These new copolyesters can also be used for microencapsulation, e.g., of cells or drugs, to produce cell growth matrices, and to produce biomedical materials such as sutures, implants, and drug delivery vehicles.

Other Embodiments

As described in Shi et al., *Polymer Preprints, Am. Chem Soc.*, 36(1):430-432 (April 1995), *A. eutrophus* grown on fructose resulted in the formation of P3HB with a high M_n. When PEG-200 was added to the culture media, the P3HB M_n decreased significantly. For example, the addition of 0.2% PEG decreased P3HB M_n by a factor of 2. Further increases in PEG concentration up to 10% resulted in decreased P3HB molecular weight by a factor of about 10.

Copolyester blends including component polymers having repeat unit structures other than 3HB and 4HB can also be made according to the invention. For example, component polymer repeat unit structures of 3-

hydroxyvalerate, 3-hydroxyhexanoate, 3-hydroxy-4-pentenoate, and 3-hydroxypropionate, can be prepared using the corresponding acids, e.g., 3-hydroxyvaleric acid, 3-hydroxyhexanoic acid, 3-hydroxy-4-pentenoic acid, and 3-hydroxypropionic acid, as carbon sources.

In addition, other carbon sources can be used to form 3HB and 4HB repeat unit structures. For example, 1,4-butane diol and 1,6-hexane diol can be used as carbon sources to produce 4HB repeat unit structures. Thus, the addition of PEG to polymer production media containing these carbon sources will also cause an increase in the 4HB mol percentage compared to fermentation without the added PEG.

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, that the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

Claims

1. A method for producing a polyhydroxyalkanoate (PHA) having a controlled molecular weight, said method comprising

culturing a PHA-producing microorganism in a polymer production medium under conditions that allow the microorganism to produce a PHA, and

adding polyethylene glycol (PEG) to the polymer production medium in an amount sufficient for the microorganism to produce a PHA having a molecular weight that is decreased relative to the molecular weight of a PHA produced by the same microorganism under the same growth conditions without PEG.

2. A method of claim 1, wherein the PHA-producing microorganism is an *Alcaligenes* bacterium.

3. A method of claim 2, wherein the PHA-producing microorganism is *Alcaligenes eutrophus*, and wherein PEG is added to the polymer production medium at a concentration of 0.25 to 10.0 percent (weight/volume).

4. A method of claim 2, wherein the PHA-producing microorganism is *Alcaligenes latus*, and wherein PEG is added to the polymer production medium at a concentration of up to 6.0 percent (weight/volume).

5. A method for incorporating 3-hydoxyvalerate (3HV) repeat units into a polyhydroxyalkanoate (PHA) using a non-3HV carbon source, said method comprising

culturing a PHA-producing microorganism in a polymer production medium containing a non-3HV carbon source under conditions that allow the microorganism to produce a PHA, and

adding polyethylene glycol (PEG) to the polymer production medium in an amount sufficient for the microorganism to produce a PHA comprising 3HV.

6. A method of claim 5, wherein the non-3HV carbon source is 4-hydroxybutyric acid or 4-hydroxybutyrate.

7. A method of claim 5, wherein PEG is added to the polymer production medium at a concentration of from 1 to 4 percent (weight/volume).

8. A method for producing a polyhydroxyalkanoate (PHA) comprising a copolyester blend of at least two component polymers wherein each polymer represents at least 30 percent by weight of the total blend, each component polymer is composed of at least 70 percent of a specific repeat unit structure, and the major repeat unit structure in each component polymer is different, said method comprising

culturing a PHA-producing microorganism in a polymer production medium containing a carbon source under conditions that allow the microorganism to produce a PHA, and

adding polyethylene glycol (PEG) to the polymer production medium in an amount sufficient for the microorganism to produce a PHA comprising a copolyester blend.

9. A method of claim 8, wherein PEG is added at a concentration of 4 percent (weight/volume).

10. A method of claim 8, wherein the carbon source is 4-hydroxybutyrate.

11. A method for producing a polyhydroxyalkanoate-polyethylene glycol (PHA-PEG) diblock copolymer in which the carboxyl terminus of a PHA chain segment is covalently linked by an ester bond to a PEG chain segment, said method comprising

culturing a PHA-producing microorganism in a polymer production medium under conditions that allow the microorganism to produce a PHA, and

adding polyethylene glycol (PEG) to the polymer production medium in an amount sufficient for the microorganism to produce a PHA-PEG diblock copolymer.

12. A method of claim 11, wherein the polymer production medium comprises glucose as the carbon source, and wherein the microorganism is *Alcaligenes latus*.

13. A method of claim 12, wherein PEG is added at a concentration of up to 6 percent (weight/volume).

14. A method of claim 12, wherein the PHA chain segment contains only P3HB repeat units.

15. A method of claim 11, wherein the polymer production medium comprises 4-hydroxybutyric acid as the carbon source, and wherein the microorganism is *Alcaligenes eutrophus*.

16. A method of claim 15, wherein PEG is added at a concentration of 4 percent (weight/volume).

17. A method of claim 15, wherein the diblock copolymer comprises a majority of 4HB repeat units.

18. A method for increasing the 4-hydroxybutyrate (4HB) mol percent in a polyhydroxyalkanoate (PHA), said method comprising

culturing a PHA-producing microorganism in a polymer production medium containing 4-hydroxybutyric acid as a carbon source under conditions that allow the microorganism to produce a PHA, and

adding polyethylene glycol (PEG) to the polymer production medium in an amount sufficient for the microorganism to produce a PHA of increased 4HB mol percent.

19. A method of claim 18, wherein PEG is added to the polymer production medium at a concentration of 1 percent (weight/volume).

20. A method of claim 18, wherein PEG is added to the polymer production medium at a concentration of 2 percent (weight/volume).

21. A polyhydroxyalkanoate (PHA) copolyester blend comprising first and second polymers each comprising at least 30 percent by weight of said blend, wherein said first polymer comprises at least 70 mol percent of a first repeat unit structure, said second polymer comprises at least 70 mol percent of a second repeat unit structure, and wherein said first and second repeat unit structures are different.

22. A copolyester blend of claim 21, wherein said first repeat unit structure is 3-hydroxybutyric acid.

23. A copolyester blend of claim 21, wherein said first polymer comprises at least 90 mol percent of a first repeat unit structure.

24. A copolyester blend of claim 21, wherein said second repeat unit structure is 4-hydroxybutyrate.

25. A copolyester blend of claim 21, wherein said second polymer comprises at least 80 mol percent of a second repeat unit structure.

26. A polyhydroxyalkanoate-polyethylene glycol diblock (PHA-PEG) copolymer comprising a first chain of PHA repeat units and a second chain of PEG repeat units, wherein the second chain of PEG repeat units is covalently bound via an ester bond to a carboxy terminal end of the first chain of PHA repeat units.

27. A PHA-PEG diblock copolymer of claim 26, wherein said first chain comprises poly-3-hydroxybutyrate, and said second chain comprises an average of 5 PEG repeat units.

28. A PHA-PEG diblock copolymer of claim 27, wherein said first chain comprises an average of 220 PHA repeat units.

29. A PHA-PEG diblock copolymer of claim 26, wherein said first chain comprises at least 80 mol percent of 4-hydroxybutyrate, and said second chain comprises an average of 5 PEG repeat units.

30. A PHA-PEG diblock copolymer of claim 29, wherein said first chain comprises an average of 435 PHA repeat units.

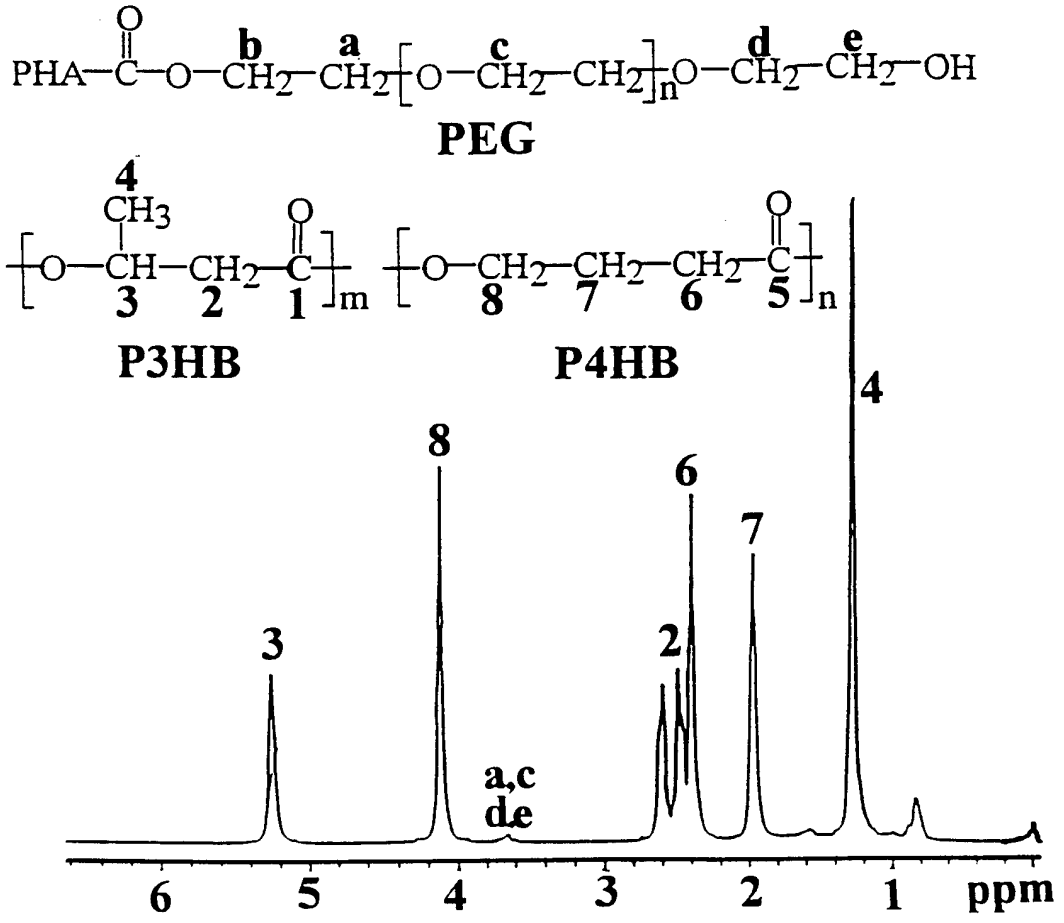

PHA—C(=O)—O—CH2—CH2—[O—CH2—CH2]n—O—CH2—CH2—OH
b a c d e
PEG
4
CH3
P3HB
P4HB
3 2 1 8 7 6 5
8
6
7
4
3
2
a,c
d,e
6 5 4 3 2 1 ppm

FIG. 1

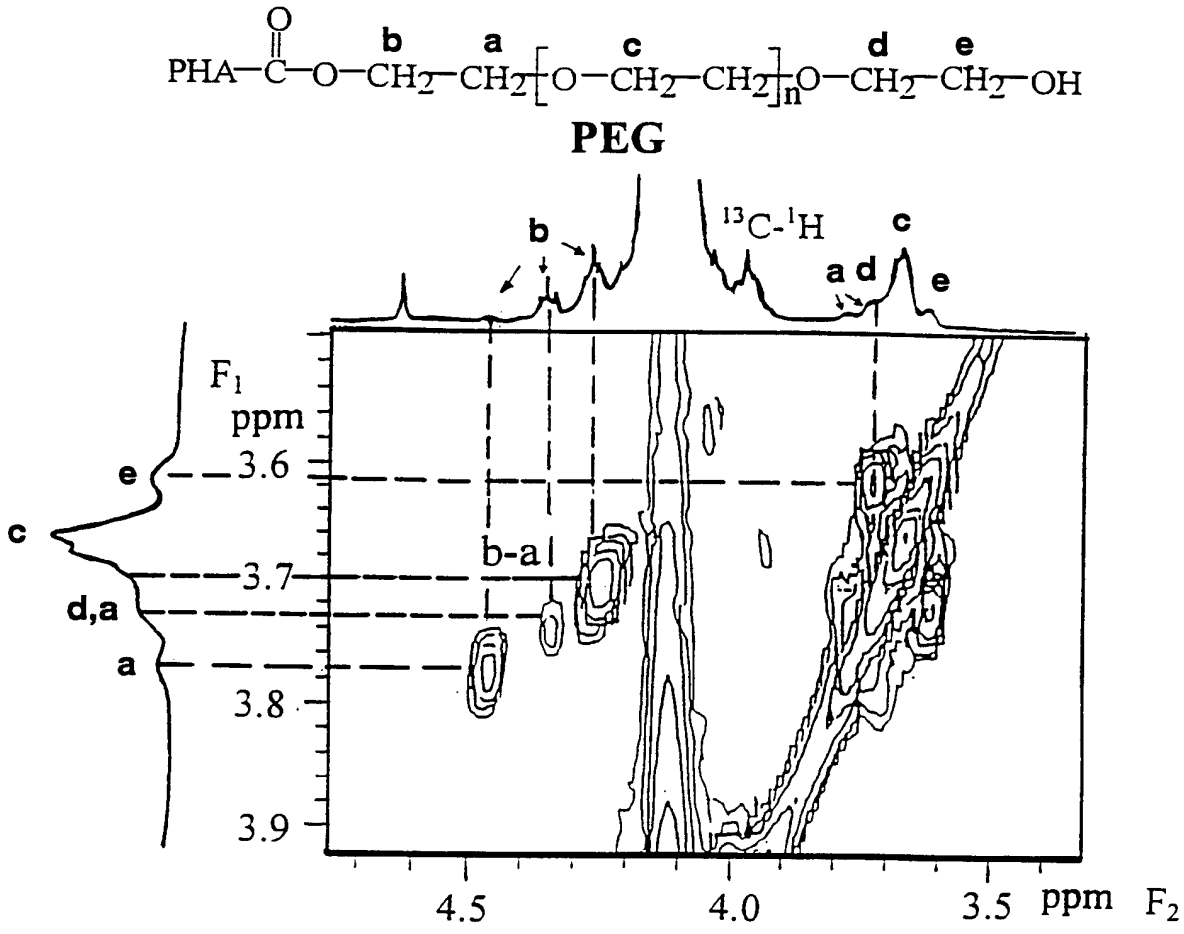

PHA—C—O—CH2—CH2—[O—CH2—CH2]n—O—CH2—CH2—OH
b a c d e
PEG
13C-1H
b
c
a d
e
F1
ppm
3.6
3.7
3.8
3.9
e
c
d,a
a
b-a
4.5
4.0
3.5 ppm F2

FIG. 2

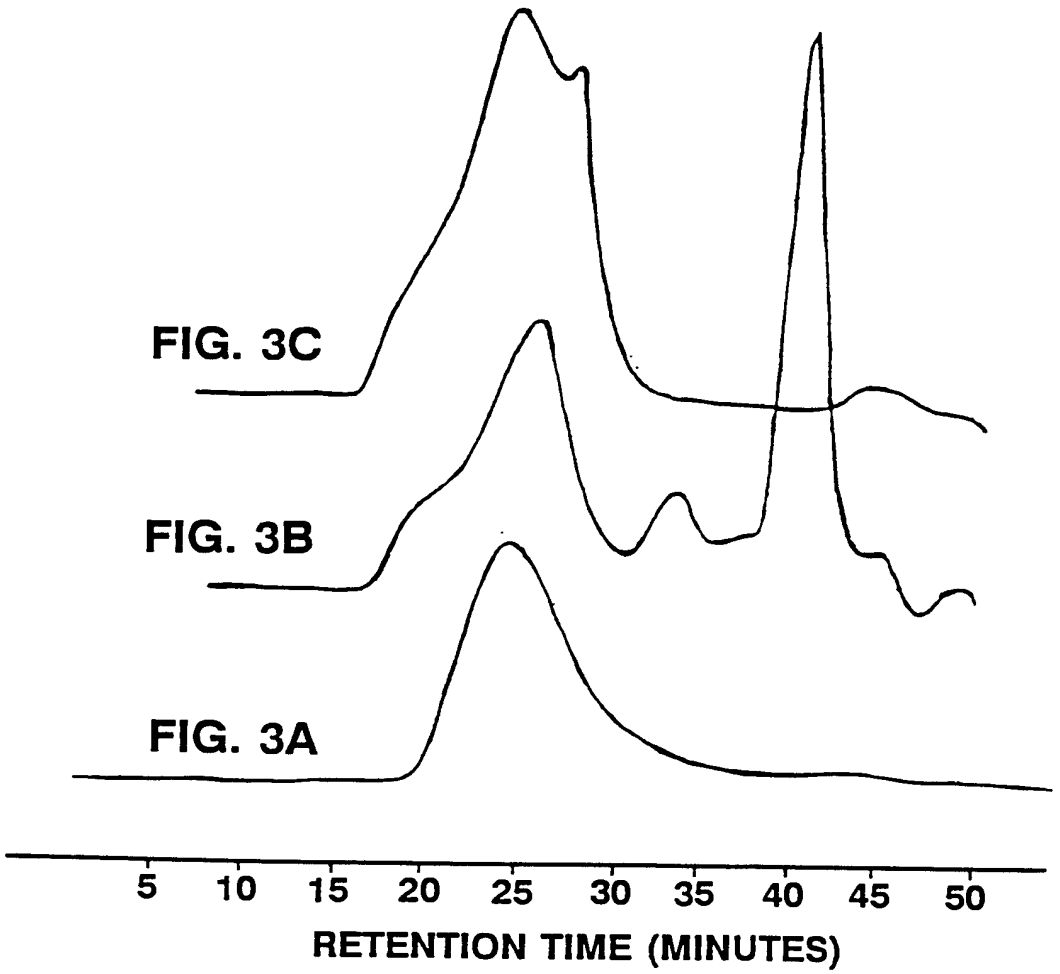


FIG. 3C
FIG. 3B
FIG. 3A
5
10
15
20
25
30
35
40
45
50
RETENTION TIME (MINUTES)

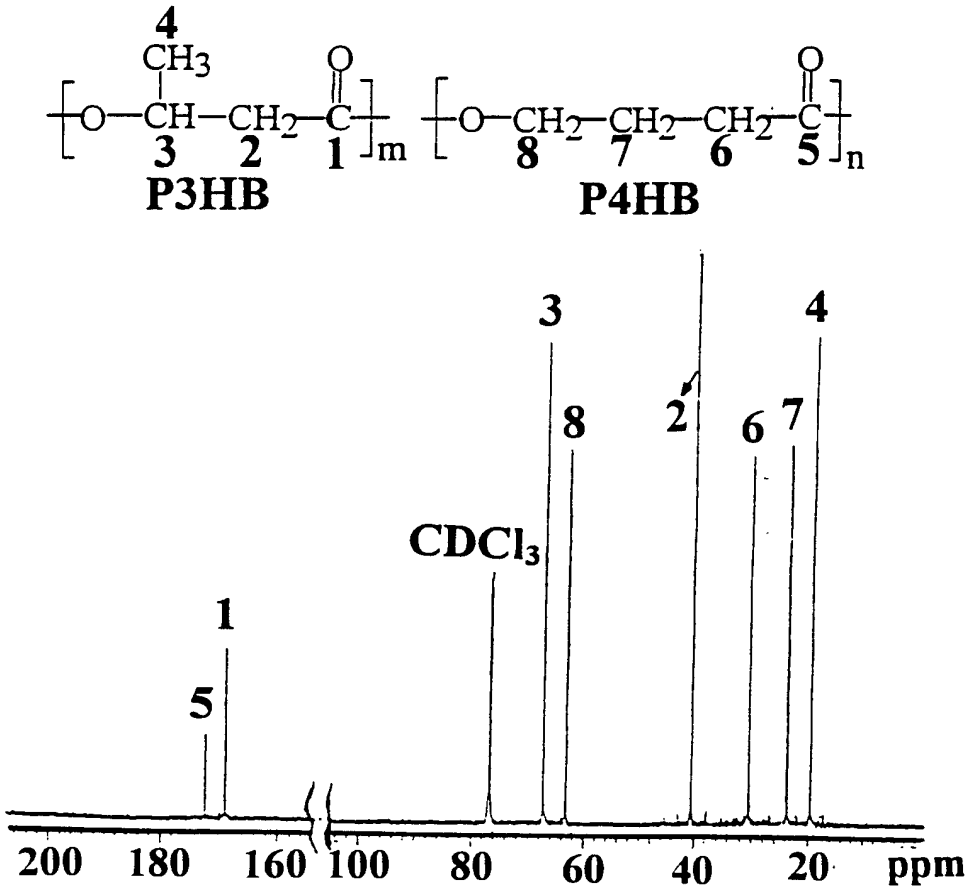

4
CH3
O
O
O
CH
CH2
C
CH2
CH2
CH2
C
3
2
1
m
8
7
6
5
n
P3HB
P4HB
3
4
8
2
6
7
CDCl3
1
5
200
180
160
100
80
60
40
20
ppm

FIG. 4

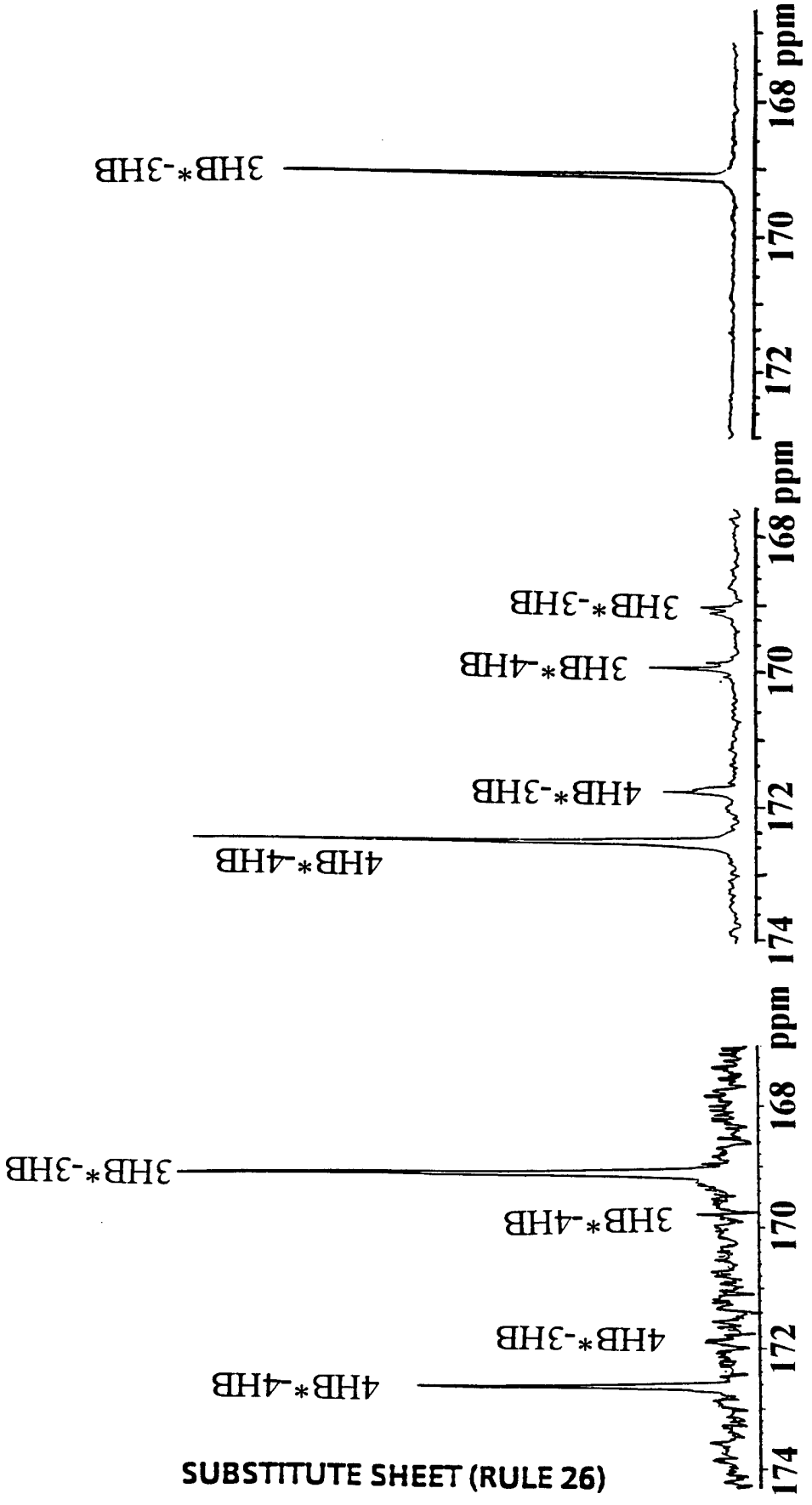
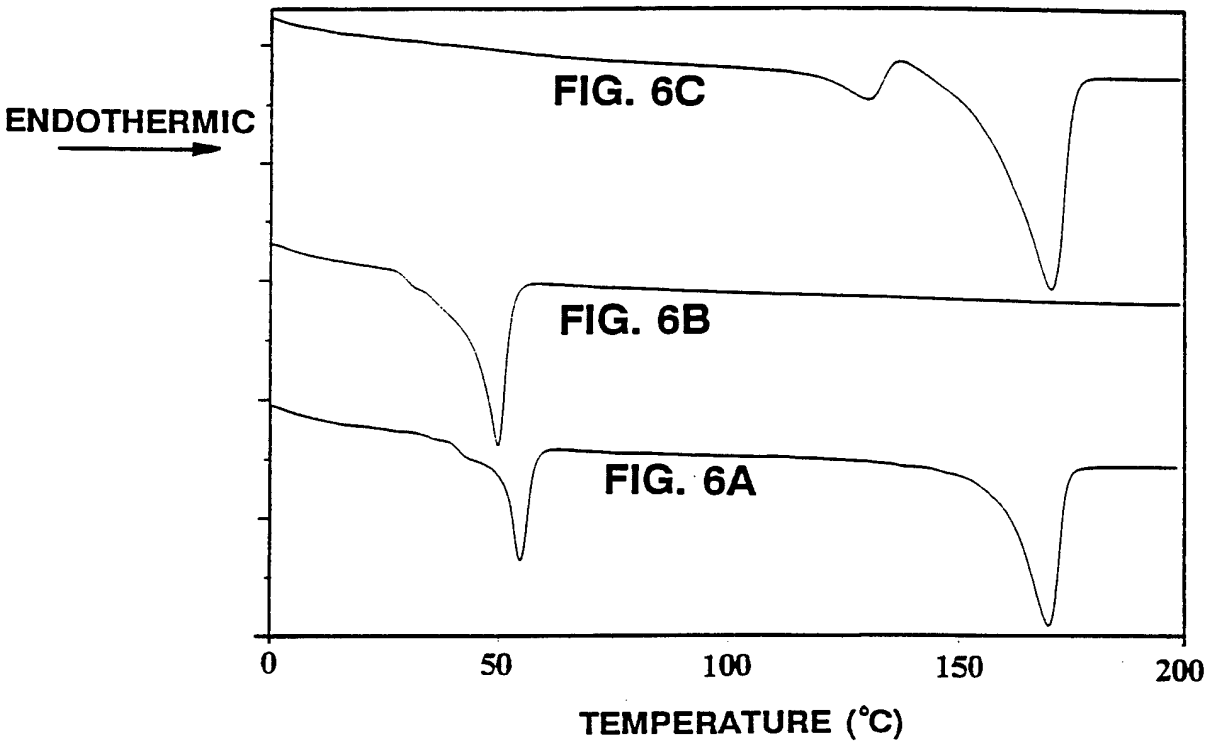


FIG. 5A

FIG. 5B

FIG. 5C


ENDOTHERMIC
FIG. 6C
FIG. 6B
FIG. 6A
0
50
100
150
200
TEMPERATURE (°C)

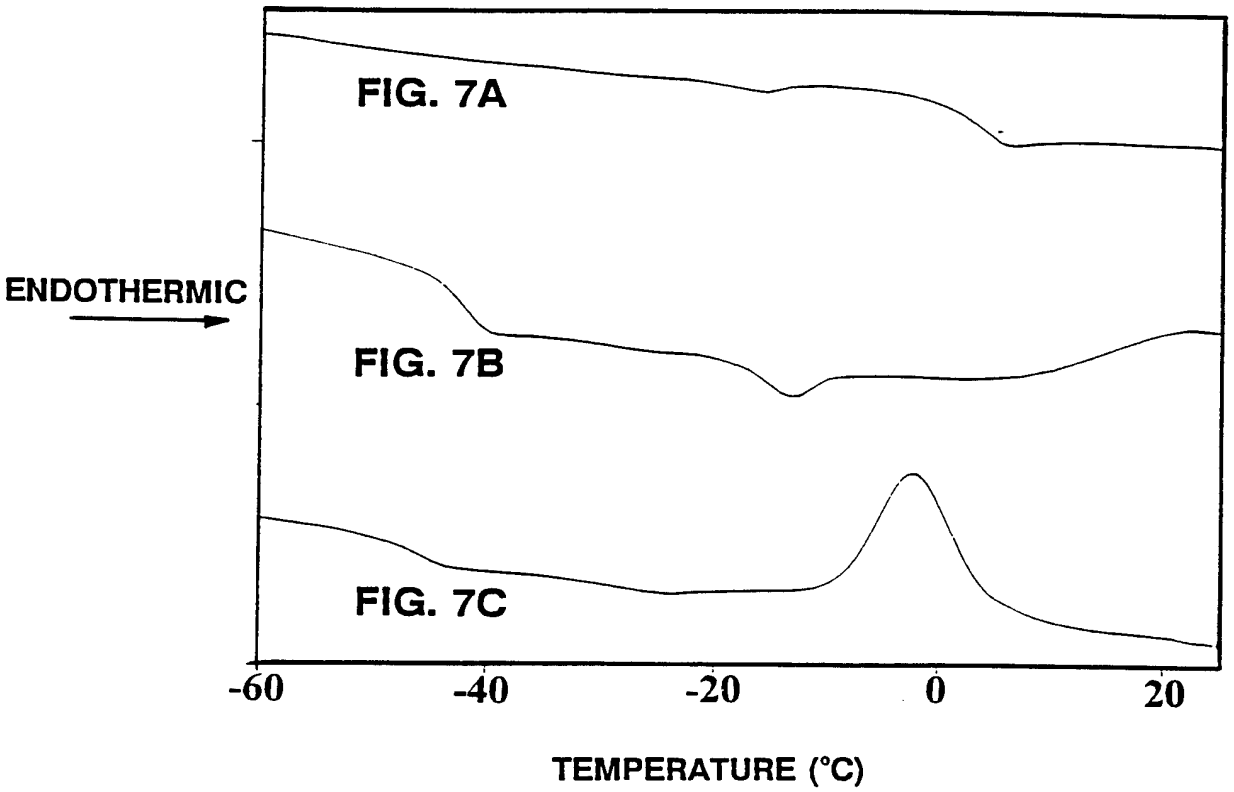


FIG. 7A
FIG. 7B
FIG. 7C
ENDOTHERMIC
-60
-40
-20
0
20
TEMPERATURE (°C)

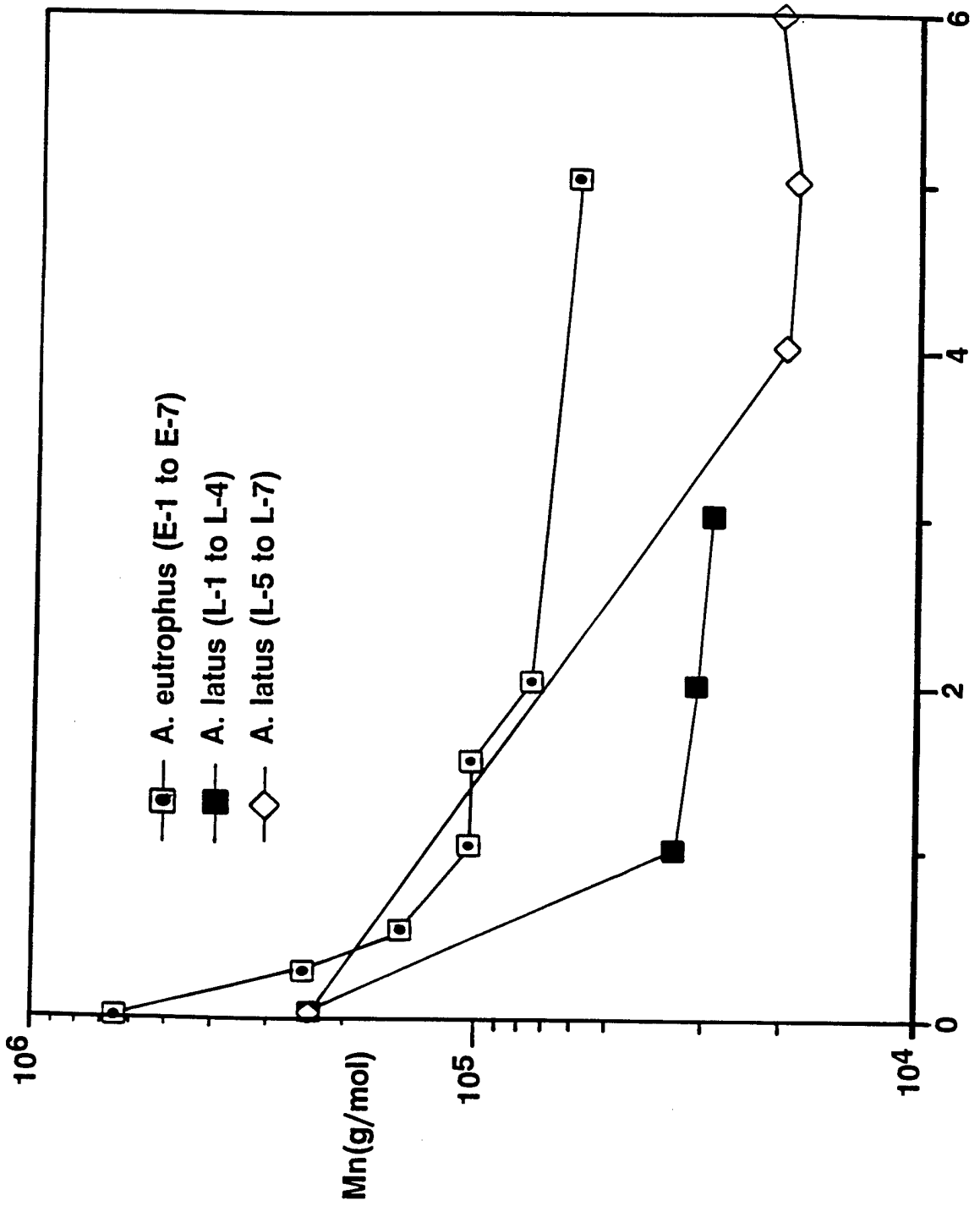

A. eutrophus (E-1 to E-7)
A. latus (L-1 to L-4)
A. latus (L-5 to L-7)
Mn(g/mol)
10^6
10^5
10^4
0
2
4
6
% PEG-200

FIG. 8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/10396

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C08G 63/00; C12P 7/12, 7/62; C12N 1/12
US CL :435/135, 252.1, 280, 822, 829; 528/354, 361
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/135, 252.1, 280, 822, 829; 528/354, 361

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US, A, 4,477,654 (HOLMES ET AL) 16 October 1984, see entire disclosure.	1-30
A	US, A, 5,200,332 (YAMANE ET AL) 06 April 1993, see entire disclosure.	1-30
Y	US, A, 5,225,227 (YALPANI) 06 July 1993, see entire disclosure.	1-30
A	US, A, 5,264,546 (ANDERSON ET AL) 23 November 1993, see entire disclosure.	1-30
A	US, A, 5,344,769 (WITHOLT ET AL) 06 September 1994, see entire disclosure.	1-30
A	US, A, 5,395,919 (LEE ET AL) 07 March 1995, see entire disclosure.	1-30

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 12 NOVEMBER 1995	Date of mailing of the international search report 12 DEC 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer DR. HERBERT J. LILLING Telephone No. (703)308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/10396

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US, A, 5,434,062 (GROLEAU ET AL) 18 July 1995, see entire disclosure.	1-30
X	Chemical Abstracts, Volume 117, Number 24, 1995, abs no. 234736f Kleinke et al, "Melt polymerization of polyhydroxyalkanoates with compounds bearing at least two reactive groups, e.g., acid and/or hydroxyl groups" Abstract of EP 491,171 issued 24 June 1992, see entire disclosure.	26
---		---
Y		1-25, 27-30